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ZnT5 Variant B Is a Bidirectional Zinc Transporter and Mediates Zinc Uptake in Human Intestinal Caco-2 Cells*

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Zinc is an essential micronutrient, so it is important to elucidate the molecular mechanisms of zinc homeostasis, including the functional properties of zinc transporters. Mammalian zinc transporters are classified in two major families: the SLC30 (ZnT) family and the SLC39 family. The prevailing view is that SLC30 family transporters function to reduce cytosolic zinc concentration, either through efflux across the plasma membrane or through sequestration in intracellular compartments, and that SLC39 family transporters function in the opposite direction to increase cytosolic zinc concentration. We demonstrated that human ZnT5 variant B (ZnT5B (hZTL1)), an isoform expressed at the plasma membrane, operates in both the uptake and the efflux directions when expressed in *Xenopus laevis* oocytes. We measured increased activity of the zinc-responsive metallothionein 2a (MT2a) promoter when ZnT5b was coexpressed with an MT2a promoter-reporter plasmid construct in human intestinal Caco-2 cells, indicating increased total intracellular zinc concentration. Increased cytoplasmic zinc concentration mediated by ZnT5B, in the absence of effects on intracellular zinc sequestration by the Golgi apparatus or endoplasmic reticulum, was also confirmed by a dramatically enhanced signal from the zinc fluorophore Rhodzin-3 throughout the cytoplasm of Caco-2 cells overexpressing ZnT5B at the plasma membrane when compared with control cells. Our findings demonstrate clearly that, in addition to mediating zinc efflux, ZnT5B at the plasma membrane can function to increase cytoplasmic zinc concentration, thus indicating a need to reevaluate the current paradigm that SLC30 family zinc transporters operate exclusively to decrease cytosolic zinc concentration.

Zinc is essential to a wide range of cellular processes through functions that include playing a critical structural role in many proteins, including approximately 1,000 human zinc finger transcription factors, and also acting as a catalytic component in several hundred human enzymes (1). Cell zinc status must be, therefore, tightly regulated.

Membrane transporters that mediate cellular zinc uptake and efflux as well as intracellular zinc sequestration play a central role in cellular zinc homeostasis, but functional characterization of many of these transport proteins remains poor. Cloned mammalian cDNAs that encode proteins with either demonstrated or assumed zinc transport activity fall into one of two main families, classified as SLC30 and SLC39 (2, 3). SLC30 family zinc transporters (ZnT transporters in mammalian systems) have been shown to reduce cytosolic zinc concentration by promoting zinc efflux from the cell or transport into intracellular compartments (3). SLC39 family transporters have been shown to function to increase cytosolic zinc concentration, transporting extracellular zinc into the cell across the plasma membrane or intracellular zinc into the cytosol from intracellular compartments (2). Researchers have studied the functional properties of zinc transporters belonging to both families through a number of different methodologies that can be divided broadly into two main approaches: (i) direct measurement of transport function and (ii) determination of the effects of transporter expression on cellular responses to zinc (*i.e.* indirect measurement).

Most evidence relating to the function of mammalian SLC30 family transporters is indirect. For example, expression of ZnT1 or ZnT2 rescued a mutant baby hamster kidney cell line from zinc sensitivity (4, 5). Also, ZnT3 knock-out mice failed to accumulate zinc in synaptic vesicles of neurons on which the ZnT3 protein is normally expressed (6), and transformation of ZnT4 into a mutant zinc-sensitive yeast strain complemented a mutation in *ZRC1*, a gene homologous to the mammalian SLC30 family transporters (7).

Direct evidence for zinc transport function within the SLC30 family includes increased zinc efflux and uptake in baby hamster kidney cells expressing ZnT1 (5), ^{65}Zn uptake into Golgi-enriched vesicles from Hela cells transfected with ZnT5 (8), and ^{65}Zn uptake across the plasma membrane of *Xenopus* laevis oocytes expressing ZnT5 (9). These apparently different reported functional activities of ZnT5 may correspond with two splice variants of the *SLC30A5* gene. The sequences reported (8, 9) differ at their N- and C-terminal regions, corresponding with the use of different 5′ and 3′ exons (10). The ZnT5 splice variants adopted different subcellular localizations

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when expressed as fusions to GFP[2] from the corresponding transgenes introduced into Chinese hamster ovary cells. Variant A was expressed in the Golgi apparatus, and variant B (ZnT5B) was expressed throughout the cell, including at the plasma membrane (10). Plasma membrane localization of ZnT5B, specifically localization at the apical membrane, was also observed in human intestinal Caco-2 cells (9), and we also reported previously localization of ZnT5 to the apical enterocyte membrane in human small intestine, using an antibody that may potentially recognize both splice variants (11).

The aim of the present study was to examine in more detail the functional properties of ZnT5B, the isoform expressed at the plasma membrane. We present additional data confirming our earlier observations that ZnT5B expressed in *X. laevis* oocytes functions to increase zinc uptake (9) but also show function in the opposite direction in the same system. In addition, we show increased zinc-responsive reporter gene expression in Caco-2 cells in which ZnT5B was overexpressed, indicative of increased zinc uptake across the plasma membrane. We also show increased intracellular zinc content in cells overexpressing ZnT5B and demonstrate that these effects of ZnT5B are not related to increased accumulation of zinc in the Golgi apparatus or endoplasmic reticulum. The observations bring into question the prevailing view that SLC30 family transporters operate exclusively to reduce cytosolic zinc and demonstrate clearly a role for ZnT5B in cellular zinc uptake.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Plasmid pCR2.1-ZnT5B, for *in vitro* transcription of ZnT5B, was generated as described previously (10). Plasmid pCDNA3.1-ZnT5B-*myc*, for *in vitro* transcription of ZnT5B with a C-terminal *myc* epitope tag, was generated by subcloning the ZnT5 insert obtained by restriction digestion of pCR2.1-ZnT5B with EcoRI into the plasmid pCDNA3.1/*myc*-HisA (Invitrogen). Plasmid pEGFP-ZnT5B, for expression of ZnT5B as an N-terminal fusion to GFP in Caco-2 cells, was generated as described previously (10). Plasmid pCDNA3.1-ZnT5B, for expression of ZnT5B in Caco-2 cells, was generated by restriction digestion of pEGFP-ZnT5B with ApaI and XhoI followed by ligation into the plasmid pCDNA3.1/Zeo (Invitrogen). The plasmid pBlue-MT2a, including the *Escherichia coli* β-galactosidase reporter gene downstream of the human MT2a promoter (from −358 to +40), was generated as described previously (12).

In Vitro Transcription of ZnT5B RNA—Plasmids pCR2.1-ZnT5B and pCDNA3.1-ZnT5-*myc* were linearized 3′ of the ZnT5 insert using the restriction endonuclease SstI. RNA was transcribed from the T7 promoter upstream of the ZnT5B cDNA using the mMessage mMachine kit (Ambion) following the manufacturer's instructions.

Measurement of ZnT5B Activity in X. laevis Oocytes—*X. laevis* oocytes were prepared and injected with ZnT5B RNA or water as described previously (9). Zinc uptake into oocytes was measured by incubating groups of 8–10 oocytes for 30 min in 300 μl of uptake buffer (100 mM NaCl, 1 mM ascorbic acid, 10 mM HEPES, pH 7.0) plus 12 μM $ZnCl_2$ including 6.4 μCi/ml $^{65}Zn^{2+}$ (PerkinElmer Life Sciences). Oocytes were washed three times in uptake buffer before measuring $^{65}Zn^{2+}$ incorporation into single oocytes as γ emission. Measurement of zinc efflux was based on a method described previously (13). Fifty nanoliters of $^{65}ZnCl_2$ at a specific activity of 6.4 μCi/ml were injected into the cytoplasm of individual oocytes. After a 30-min recovery period, each oocyte was transferred into a separate vial containing 0.5 ml of modified Barth's medium (9). The efflux of radioactive ^{65}Zn was allowed to continue for 20 min, after which time the buffer was transferred to a counting vial for measurement of γ emission, and 0.5 ml of fresh buffer were added. This process was repeated for 100 min. The amount of radioactivity that remained in each oocyte was also determined. Tracer efflux was assessed from plots of $-\ln(C_t/C_0)$ against time, where C_0 and C_t are the levels of activity (dpm) in the cell at the start and end, respectively, of each experimental period (obtained by summation of residual oocyte radioactivity and sequential radioactive losses to vials by tracer efflux).

Localization of ZnT5B in X. laevis Oocyte—Oocytes injected with RNA transcribed *in vitro* from plasmid pCDNA3.1-ZnT5B-*myc* were fixed at 4 °C for 1 h in 4% (w/v) paraformaldehyde, prepared in PBS. After washing in PBS, oocytes were incubated at 4 °C in a 30% sucrose solution overnight. Oocytes were frozen rapidly in OCT medium (VWR International), and sections were cut to a thickness of ~15 μm using a Shandon Cryotome. Sections were mounted on slides and incubated for 20 min in 200 μl of blocking solution (PBS containing 10% fetal calf serum). The blocking solution was removed, and the slides were incubated in the dark for 1 h with 100 μl of PBS, 10% fetal calf serum containing FITC-conjugated anti-*myc* antibody (Invitrogen; 1:500 dilution of antibody). After incubation, slides were washed twice in PBS. Sections were then mounted in Vectashield mounting medium (Vector Laboratories Ltd.), and the pattern of fluorescence was visualized by fluorescence microscopy (Leica DMBRE).

Transfection of Caco-2 Cells and Measurement of Reporter Gene Activity—Human intestinal Caco-2 cells were cultured routinely as described previously (9). For transfection, Caco-2 cells were seeded into 12-well plates at 3.5×10^5 cells/well. Cells were transfected 24 h after seeding using Genejammer reagent (Stratagene) following the manufacturer's instructions and using a ratio of DNA:transfection reagent of 2 μg:4.5 μl. After 24 h, transfected cells were incubated in serum-free medium, with or without the addition of 100 μM $ZnCl_2$ or 100 μM $CuSO_4$ plus 1 mM ascorbate, for a further 24 h. β-Galactosidase activity was measured in cell lysates using the substrate chlorophenol red-β-D galactopyranoside (Sigma), as described previously (12). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories) with bovine serum albumin (Sigma), as the standard.

Visualization of Intracellular Zinc Concentration Using Rhodzin-3—Caco-2 cells were seeded at a density of 5×10^5 cm^2 onto glass coverslips. Twenty-four hours after seeding, cells were transfected with pEGFP-ZnT5B or vector only (pEGFPN; Clontech). After a further 24 h, cells were loaded with the zinc fluorophore Rhodzin-3 AM (5 μM; Invitrogen) for

[2] The abbreviations used are: GFP, green fluorescent protein; ZnT5B, ZnT5 variant B; PBS, phosphate-buffered saline; MT2a, metallothionein 2a; FITC, fluorescein isothiocyanate.

30 min at room temperature. Cells were washed with PBS and left at 37 °C for 1 h to allow de-esterification. Cells were treated with 100 μM $ZnCl_2$ in serum-free medium for a further 24 h prior to fixing for 1 h in 4% (v/v) paraformaldehyde, prepared in PBS. Cells were mounted in Vectashield mounting medium (Vector Laboratories Ltd.), and the pattern of fluorescence was visualized by confocal laser scanning microscopy (Leica TCS NT).

Data Analysis—Data are presented as mean ± S.E. Statistical analysis was carried out using the Microsoft SPSS package. Differences were considered significant at $p < 0.05$.

RESULTS

We assayed directly the functional activity of ZnT5B by measuring the flux of the radiotracer $^{65}Zn^{2+}$ across the plasma membrane of *X. laevis* oocytes injected with ZnT5B RNA. The uptake of $^{65}Zn^{2+}$, supplied at 12 μM, into oocytes injected with ZnT5B cRNA was significantly greater (~4-fold) than into uninjected controls (Fig. 1*A*). For measurement of zinc efflux, oocytes were injected with 50 nl (5 μCi/μl) of $^{65}ZnCl_2$. The efflux of $^{65}Zn^{2+}$ was increased in oocytes injected with ZnT5B RNA when compared with water-injected controls (Fig. 1*B*). The rate constant for zinc efflux in this experiment was calculated as the slope of progressive tracer loss ($\ln (C_t/c_o)$) with time and was 2.45 ± 0.4 ($\times 10^{-3}$)/min for ZnT5-injected oocytes and 1.15 ± 0.2 ($\times 10^{-3}$)/min for controls ($n = 8$ cells each, $p < 0.0001$ by one way analysis of variance followed by Dunnett's multiple comparisons test). Increasing the extracellular Zn^{2+} concentration to 1 mM had no effect on $^{65}Zn^{2+}$ efflux (Fig. 1*B*), indicating that ZnT5B can mediate zinc efflux against a concentration gradient. To establish the location of the heterologously expressed ZnT5B in *X. laevis* oocytes, cells were injected with a C-terminal *myc*-tagged ZnT5B construct, and the protein was detected in sections of paraformaldehyde-fixed oocytes using a FITC-labeled anti-*myc* antibody. Immunofluorescence at the plasma membrane in oocytes injected with ZnT5B-*myc* cRNA was observed and was clearly not visible in water-injected controls (Fig. 1*C*), demonstrating localization at the plasma membrane.

To determine the effect of increased ZnT5 expression on intracellular zinc concentration in a physiologically relevant cell type, we measured the effect of heterologous overexpression of ZnT5B, in the vector pCDNA3.1, on the activity of a zinc-activated reporter gene, comprising the *E. coli* β-galactosidase coding sequence downstream of the zinc-activated human MT2a promoter. Co-expression of ZnT5 (from plasmid pCDNA3.1-ZnT5B) with the reporter construct (plasmid pBlue-MT2a) increased significantly β-galactosidase activity (~4-fold) in serum-free medium with and without the addition of 100 μM extracellular zinc when compared with cells co-transfected with pBlue-MT2a plus vector pCDNA3.1 only (no ZnT5 insert), indicating increased transcription from the zinc-responsive MT2a promoter and so increased intracellular zinc concentration (Fig. 2*A*). To exclude the possibility that the effect of ZnT5B overexpression on MT2A promoter activity was the result of any change in intracellular Cu^{2+} concentration or distribution, we demonstrated that the reporter gene activity was unaffected by changes in Cu^{2+} availability in this experimental system (Fig. 2*B*).

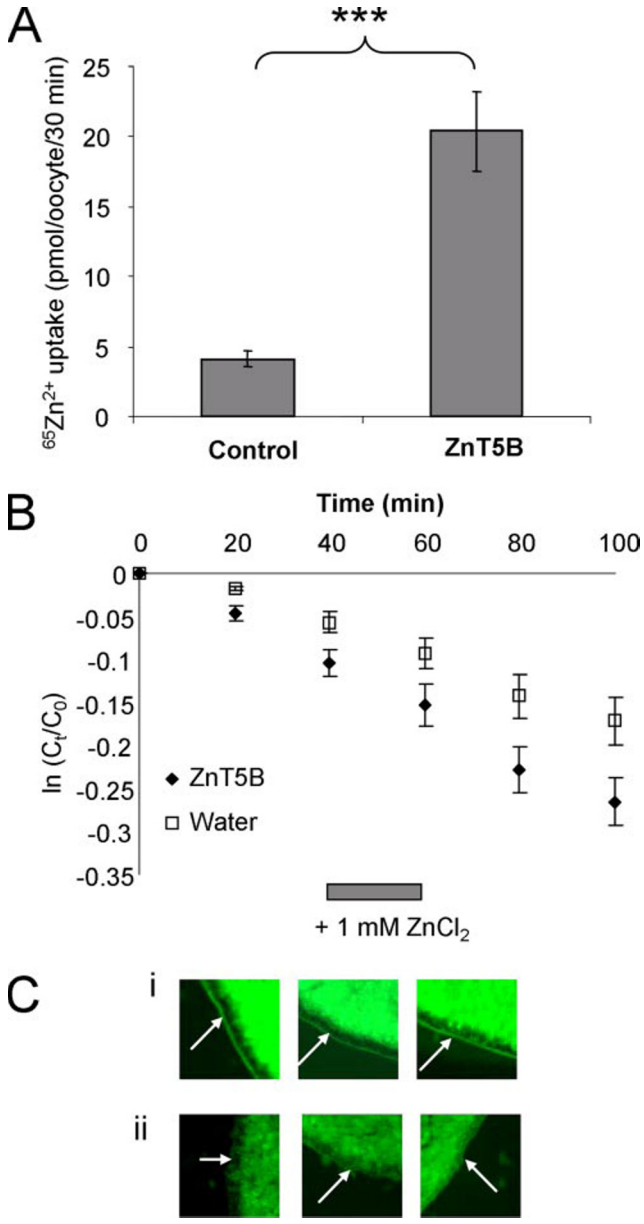

FIGURE 1. **ZnT5B-mediated transmembrane zinc flux in *X. laevis* oocytes.** *A* shows that uptake of $^{65}Zn^{2+}$ over 30 min from a solution containing 12 μM $ZnCl_2$ by oocytes expressing ZnT5B (transcribed *in vitro* from plasmid pCR2.1-ZnT5B) or by uninjected controls, as indicated. Data are expressed as mean ± S.E. for $n = 8$–10 oocytes. ***, $p < 0.001$ by Student's *t* test. *B* shows the efflux of $^{65}Zn^{2+}$ with time from oocytes injected with ZnT5B cRNA (*filled symbols*) when compared with water-injected controls (*open symbols*). Fifty nanoliters of $ZnCl_2$ at a specific activity of 5 μCi/μl was injected into each oocyte, and efflux was followed over 100 min. Efflux in the presence of 1 mM $ZnCl_2$ (between 40 and 60 min) is indicated. C_0 and C_t are the levels of activity (dpm) in the cell at the start and end, respectively, of each experimental period (obtained by summation of residual oocyte radioactivity and sequential radioactive losses to vials by tracer efflux). Data are expressed as mean ± S.E. for $n = 8$–10. *C*, *panel i*, shows representative images of three oocytes injected with ZnT5B cDNA tagged at the C terminus with the *myc* epitope (transcribed *in vitro* from plasmid pCDNA3.1-ZnT5B-*myc*) and probed with FITC-conjugated anti-*myc* antibody (*green*) viewed using the ×200 objective lens. Specific anti-*myc* immunofluorescence is clearly visible. *C*, *panel ii*, shows representative images of three control (water-injected) oocytes probed with FITC-conjugated anti-*myc* antibody viewed using the ×200 objective lens. Anti-*myc* specific staining is absent in these controls. *Arrows* point to the plasma membrane in each case.

We determined the effects of ZnT5B overexpression, as an N-terminal fusion to GFP to allow visualization, on intracellular zinc concentration and distribution in Caco-2 cells using the

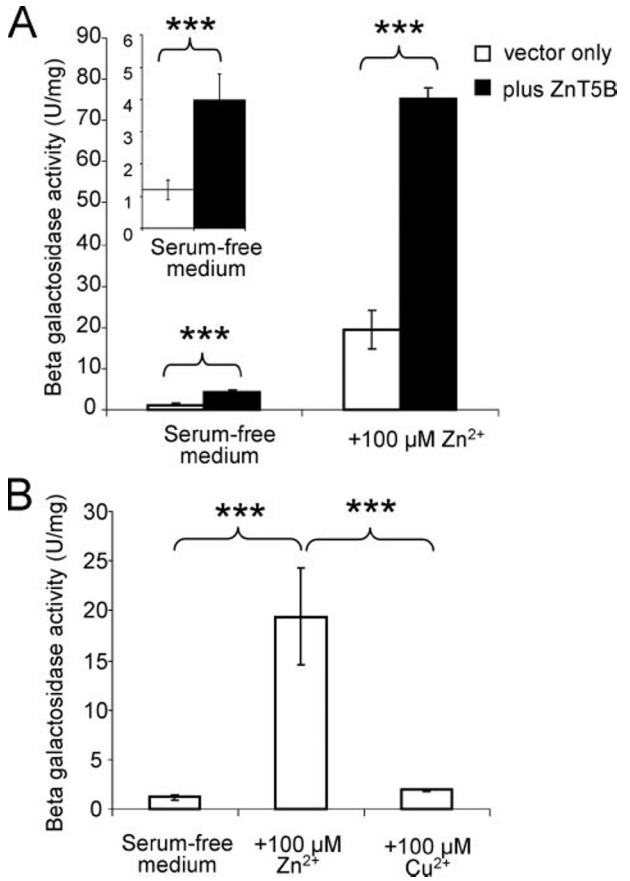

FIGURE 2. **The effect of ZnT5B expression on the activity of the zinc-activated MT2a promoter.** Data are β-galactosidase activity in lysates from Caco-2 cells co-transfected transiently with an MT2a-promoter-β-galactosidase-reporter construct (pBlue-MT2a) plus ZnT5B (pCDNA3.1-ZnT5B) or corresponding vector only (pCDNA3.1), as indicated (*A*), or transfected with pBlue-MT2a only (*B*), and maintained for 24 h in serum-free medium with or without the addition of 100 μM $ZnCl_2$ (*A* and *B*) or 100 μM $CuSO_4$ plus 1 mM ascorbate (*B*), as indicated. The *inset graph* in *A* shows the measurements made in serum-free medium plotted on an expanded scale, for clarity. Data are expressed as mean ± S.E. for $n = 3–6$. ***, $p \leq 0.001$ by one-way analysis of variance followed by Bonferroni's multiple comparisons test.

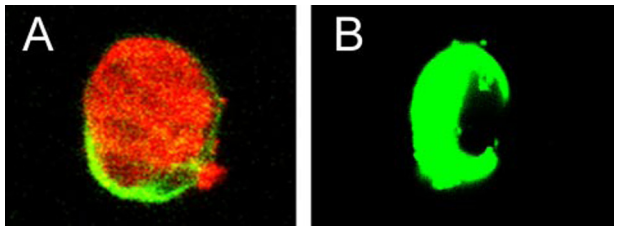

FIGURE 3. **Visualization of increased cellular zinc content in cells overexpressing ZnT5B.** Caco-2 cells were transfected with ZnT5 expressed as an N-terminal fusion to GFP (pEGFP-ZnT5B (*A*)) or with vector only (pEGFPN (*B*)). Twenty-four hours after transfection, cells were loaded with the zinc fluorophore Rhodzin-3 prior to treatment with 100 μM $ZnCl_2$ for 24 h. Cells were then fixed in 4% paraformaldehyde. GFP (shown in *green*) and Rhodzin-3 (shown in *red*) were detected by confocal laser-scanning microscopy.

zinc-activated fluorophore Rhodzin-3 (Invitrogen). Cells expressing ZnT5B as an N-terminal fusion to GFP (from plasmid pEGFP-ZnT5B) showed localization of ZnT5B to the plasma membrane along with clear cytoplasmic red/orange fluorescence, which was not visualized in cells transfected with vector pEGFPN only (Fig. 3), indicating dramatically elevated total intracellular zinc concentration in cells expressing ZnT5B at the plasma membrane.

DISCUSSION

It is becoming widely accepted that members of the SLC30 family of mammalian zinc transporters function to reduce cytosolic zinc concentration by promoting zinc efflux from the cell or intracellular zinc sequestration. The results of the current study and evidence from previous work (9) indicate clearly that this dogma should be challenged. Specifically, ZnT5B can mediate zinc uptake across the plasma membrane.

Expression of ZnT5B at the plasma membrane in *X. laevis* oocytes resulted in increased transmembrane fluxes of $^{65}Zn^{2+}$, consistent with the view that ZnT5B mediates zinc transport across the plasma membrane. Previously, we demonstrated that ZnT5B mediates Zn^{2+} uptake in the *X. laevis* oocyte (9). We now provide further confirmation of these observations using a modified uptake buffer in which ZnT5B-mediated zinc uptake at an extracellular concentration of 12 μM was greater when compared with control (4-fold higher) than was measured previously (~2-fold higher). We also now demonstrate that ZnT5B is expressed at the plasma membrane in *X. laevis* oocytes. In addition, we demonstrate the efflux of Zn^{2+} in the same expression system. Based on these observations, we suggest that ZnT5B is capable of bidirectional Zn^{2+} transport. A possible explanation for increased Zn^{2+} flux in a specific direction (uptake or efflux) in oocytes expressing ZnT5B is that it is a compensatory response to ZnT5B-mediated zinc transport in the opposite direction. Such a mechanism was suggested to explain the increase in zinc uptake as well as efflux into baby hamster kidney cells expressing ZnT1 (5). We demonstrated previously that the possibility that zinc uptake into oocytes expressing ZnT5B is compensatory for zinc efflux can be excluded by virtue of a differential effect of extracellular pH on the ZnT5B-mediated and endogenous oocyte zinc uptake mechanisms (9). The possibility that efflux was a compensatory response to zinc uptake can be excluded on the grounds that ZnT5B-stimulated efflux occurred in the absence of extracellular zinc. A major exchange mode of ZnT5B function can be excluded because $^{65}Zn^{2+}$ effluxes were not *trans*-stimulated in the presence of extracellular zinc. Bidirectional transporter-mediated transport of zinc is not without precedent and was reported in *Saccharomyces cerevisiae* for the transporter Yke4p, a member of the SLC39 family (14).

To measure ZnT5B function in a physiologically relevant cell type, the effect of increased ZnT5B expression on the intracellular zinc status of the human intestinal cell line Caco-2 was determined by measuring the activity of a zinc-stimulated reporter gene. Increased activity of the zinc-responsive MT2a promoter when ZnT5B was co-expressed in Caco-2 cells indicated increased total intracellular zinc content, altered intracellular zinc partitioning or altered status, or intracellular distribution with respect to other metals capable of activating MT expression. It is known that copper can activate metallothionein gene expression (15), but an effect on copper status and/or distribution as being responsible for the observed activation of the MT2a promoter can be excluded because extracellular Cu^{2+} did not induce an MT2a promoter response in this system. An increase in total intracellular zinc mediated by ZnT5B

expression at the plasma membrane, in the absence of effects on intracellular zinc sequestration, was confirmed by a greatly increased signal from the zinc fluorophore Rhodzin-3 throughout the cytoplasm in Caco-2 cells expressing a GFP-tagged ZnT5B transgene. There was no evidence of zinc accumulation in the Golgi apparatus, where localization of ZnT5 splice variant A (8, 10), as well as of ZnT6 (16) and ZnT7 (17), has been reported, or in the endoplasmic reticulum. It has been reported that Rhodzin-3 is a marker of mitochondrial zinc content (18), so it is feasible that the pattern of localization we observed included a component due to increased total cytoplasmic zinc concentration resulting in uptake into mitochondria.

The evident bidirectional transport capability of ZnT5B raises questions about the physiological role of this transporter. We demonstrated previously apical location of ZnT5B in Caco-2 cells (9) and detected ZnT5 at the apical membrane in human small intestine (11). An apically located intestinal zinc transporter operating in an uptake mode would facilitate the absorption of dietary zinc. Our previous observation that expression of ZnT5 was reduced in the small intestinal mucosa of human volunteers given a zinc supplement for 14 days (11) can be rationalized in terms of this being a homeostatic response to reduce the uptake of excess zinc by the transporter operating in uptake mode. An apical intestinal zinc transporter operating in efflux mode may regulate net zinc absorption to maintain homeostasis at high dietary intakes, but our data on the regulatory response of the transporter to increased zinc intake (11) appear inconsistent with such a role for ZnT5B in the intestine.

In summary, the data presented demonstrate that ZnT5 can operate in an efflux mode, in agreement with the accepted functional characteristics of zinc transporters belonging to the SLC30 family, but also has the capability to increase intracellular zinc content in the absence of any effect on zinc sequestration into specific intracellular compartments. The findings, therefore, necessitate re-evaluation of the current paradigm that SLC30 family zinc transporters operate exclusively to decrease cytosolic zinc concentration.

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